Influence of Micro-mixing on the Size of Liposomes Self-Assembled from Miscible Liquid Phases

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Abstract

Ethanol injection and variations of it are a class of methods where two miscible phases—one of which contains dissolved lipids—are mixed together leading to the self-assembly of lipid molecules to form liposomes. This method has been suggested, among other applications, for in-situ synthesis of liposomes as drug delivery capsules. However, the mechanism that leads to a specific size selection of the liposomes in solution based self-assembly in general, and in flow-focussing microfluidic devices in particular, has so far not been established. Here we report two aspects of this problem. A simple and easily fabricated device for synthesis of monodisperse unilamellar liposomes in a co-axial flow-focussing microfluidic geometry is presented. We also show that the size of liposomes is dependent on the extent of micro-convective mixing of the two miscible phases. Here, a viscosity stratification induced hydrodynamic instability leads to a gentle micro-mixing which results in larger liposome size than when the streams are mixed turbulently. The results are in sharp contrast to a purely diffusive mixing in macroscopic laminar flow that was believed to occur under these conditions. Further precise quantification of the mixing characteristics should provide the insights to develop a general theory for size selection for the class of ethanol injection methods. This will also lay grounds for obtaining empirical evidence that will enable better control of liposome sizes and for designing drug encapsulation and delivery devices.

Keywords: Liposome Synthesis, Coacervation, Ethanol Injection, Microfluidics, Hydrodynamic Stability

1. Introduction

Liposomes offer a great potential in drug delivery systems (Lasic, 1992) in terms of versatility in size and charge, ability to encapsulate hydrophilic (inside the aqueous core) and lipophilic drugs (within the lipid bilayer), relative non-toxicity compared to other carrier systems, ability to escape macrophages in the lungs (stealth liposomes) and ease of preparing covalently coupled antibodies to ensure their cell specificity i.e., targeting (Torchilin, 2005; Lasic, 1998). Liposome size and size distribution are critical parameters to control, since these influence drug dosage and the clearance rate of the drug from the body. Liposome sizes in the range 100 nm to 200 nm is most suitable for passive targeting of cancerous tissue by enhanced permission and retention mechanism (EPR) (Maeda et al., 2000; Lembo and Cavalli, 2010).

One of the ways liposome formation occurs is when of lipids molecules self-assemble from a solution phase due to changed solvent conditions, such as increase in hydrophobic costs due to the entry of water. This phenomenon may be classified under a broad principle of coacervation (Ishii et al., 1995) and ethanol injection is one such coacervation methods in which lipids dissolved in ethanol are brought into contact with water (Batzri and Korn, 1973; Kremer et al., 1977; Wagner et al., 2002) . Ethanol and water being miscible in all proportions, leads to a quick mixing of the phases resulting in induction of the lipid self-assembly into bilayers. The initial structures are disc-like membranes, which grow and close upon themselves or fuse with other discs to form vesicular compartments. The size of these lipid vesicles ranges from about 20 nm up to about a few micrometers and they may be composed of one or more concentric membranes (unilamellar and multilamellar vesicles), each with a thickness of about 4 nm (Lasic, 1993).

There is only a general understanding of the phenomenon of liposome formation in spontaneous self-assembly—formation of discs of the bilayer membrane (Lasic, 1992) which close upon to form vesicles after a critical size when the edge energy balances the bending energy (Helfrich, 1973). There has so far been no definite prediction of the size of liposomes formed in the ethanol injection methods, mainly owing to method of mixing the two phases and a wide distribution of sizes. The size prediction and control, as outlined above, is crucial from a drug-delivery point of view.

In the general class of ethanol injection methods suggested in the literature is the microfluidic method, offering a fine control on manipulation of fluid and interfaces, and in small volumes of fluids in the picoliter range. Recently, Jahn et al. (2004, 2007) introduced a microfluidic two stream hydrodynamic-focussing method (Ottino and Wiggins, 2004) which was shown to generate liposomes of varying sizes and distributions by controlling the flow rates. In this device, phospholipids dissolved in a water miscible solvent (such as ethanol) is sent through central mi-

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crochannel and water through a side microchannel in a planar geometry.

Jahn et al. (2007) suggested that an interplay between molecular diffusion of alcohol and laminar convection results in the formation of liposomes of controllable distribution. The size control was achieved in the range 50 to 150 nm by adjusting the ratio of flow rates and the over all flow rate. While the observations provided an empirical way to achieve a control of the liposome size, we do not have a quantitative model for predicting the sizes. This lack of knowledge does not confine itself to the microfluidic method alone. In the entire gamut of ethanol injection methods (Batzri and Korn, 1973; Kremer et al., 1977; Wagner et al., 2002; Ishii et al., 1995) there is no theoretical model yet that can explain the size selection or the formation of multi-lamellar liposomes in some methods.

We hypothesise that the nature of flow and mixing has a significant effect on determining the outcome of the self-assembly. To show this, we first choose a simpler configuration—core-annular two stream flow, and compare the liposome formation in this system along with other methods of mixing the two streams. We find interesting connections of the liposome size with the nature of micro-mixing.

An additional contribution in this paper is in the method of device fabrication itself. While the channel geometry suggested in Jahn et al. (2004) is easy to replicate, it requires sophisticated instruments to create the initial moulds. The channel flow also poses a theoretical difficulty in understanding the interaction of flow and diffusion, as there are no simple analytical solutions to the velocity and concentration profiles. A co-axial core-annular (or core-sheath) flow is easily amenable to simple calculations owing to the axial symmetry of the solution. Here we introduce a simple method to fabricate a co-axial hydrodynamic focussing device to synthesise liposomes following the ideas of glass-PDMS hybrid microfluidic device (Jeong et al., 2004), and a method of swelling of PDMS in an organic solvent to create channels (Verma et al., 2006). All of these can be easily reproduced in any laboratory without requirement of sophisticated instruments and environments to carry out photolithography (such as, spin-coating, mask alignment tools, gold room etc.).

We first outline the procedure to fabricate the co-axial (core-annular) flow generating device in the Methods section. Following this, we show various studies using the axial flow-focussing method—the effect of flow rates, type of mixing, lipid concentration, ethanol concentration, lipid chemistry on the size of liposomes. We then present evidence that supports our claim that it is micromixing that leads to selection of larger size liposomes formed within the micro channel.

2. Materials and Methods

2.1. Materials

The phospholipids used to synthesize the liposomes in the present method are: 1,2-dimyristoyl-sn-glycero-3-phosphocholine (14:0-DMPC); 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16:0-DPPC) (Avanti Polar Lipids), 1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1-DOPC) (Sigma Aldrich), and Hydrogenated Soy-phosphocholine (gift sample from Lipoid) is used as purchased without further purification. Ethanol (AR grade, Merck) is used to dissolve phospholipids.

All the lipids from Avanti Polar Lipids were in a solution form with chloroform (20 mg per ml) as solvent. An appropriate volume of lipid solution is taken out in a round bottom flask (RBF) and chloroform is evaporated from lipid solution using a rotary evaporator. This forms a thin film on the glass surface of the RBF. The RBF is then kept in a vacuum desiccator overnight to remove traces of chloroform. An appropriate volume of ethanol is added to the RBF to make various concentrations of the phospholipids in ethanol.

The aqueous phase used in all the experiments is Milli-Q water which has been analysed by light scattering to ensure that no extraneous particles are present. The dye used for visualisation experiments is a fountain pen ink (Camlin Royal Blue) dissolved in ethanol, which is carefully filtered through a 0.2 μm filter paper, to limit the size of particles. After filtering it was found that the solid dye particles had a unimodal distribution of sizes with mean diameter 220–250 nm.

2.2. Microfluidic device fabrication procedure

Here we describe the method to prepare the co-axial flow microfluidic device in some detail. The method is easily reproducible in any laboratory with basic chemicals and instruments. An open chamber is prepared with the help of a glass slide and strips of double sided tape as side walls. Two Borosilicate glass capillary tubes (30 mm and 90 mm in length, 1 mm outer diameter (OD) and 0.5 mm inner diameter (ID): GD-1, Narishige, Japan) are joined together using using Araldite® (which is soluble in chloroform) into a Y-shaped geometry as shown in Figure 1, and is inserted through a hole in the tape inside the chamber. Liquid Polymer polydimethyl siloxane (PDMS) (Dow corning Sylgard 184) is then poured in the chamber and left for curing at 90°C for two hours. The glass slides are removed and the now solid PDMS block is left in chloroform for two hours to allow it to swell (degree of swelling is >150%). The side glass capillary is now detachable, leaving a channel in its place. The main capillary is retracted partly up to the Y-junction, and left inside the PDMS block to serve as a hydrophilic side wall for the outlet stream. A pulled capillary (pulled to an ID of 40 μm, with a glass micropipette puller: Narishige PC-10, Japan), is then inserted through the hollow PDMS channel to go past the Y-junction through the main capillary. The length of the pulled capillary is such that the entire tube is inside the PDMS channel. Two blunt needles of appropriate size are then inserted through inlet channels up to the glass capillary. The PDMS block is then left at room temperature for the chloroform to evaporate and regain its original shape and size, now tightly holding the capillary and the needles. Since the flow usually has a small Reynolds number, the flow through the side channel attains the fully developed flow in a short distance, and can be used to hydrodynamically focus the core flow. Teflon tubing is used to connect the microchannel to the syringe (Phapal et al., 2011). Two Syringe pumps (NE1000,

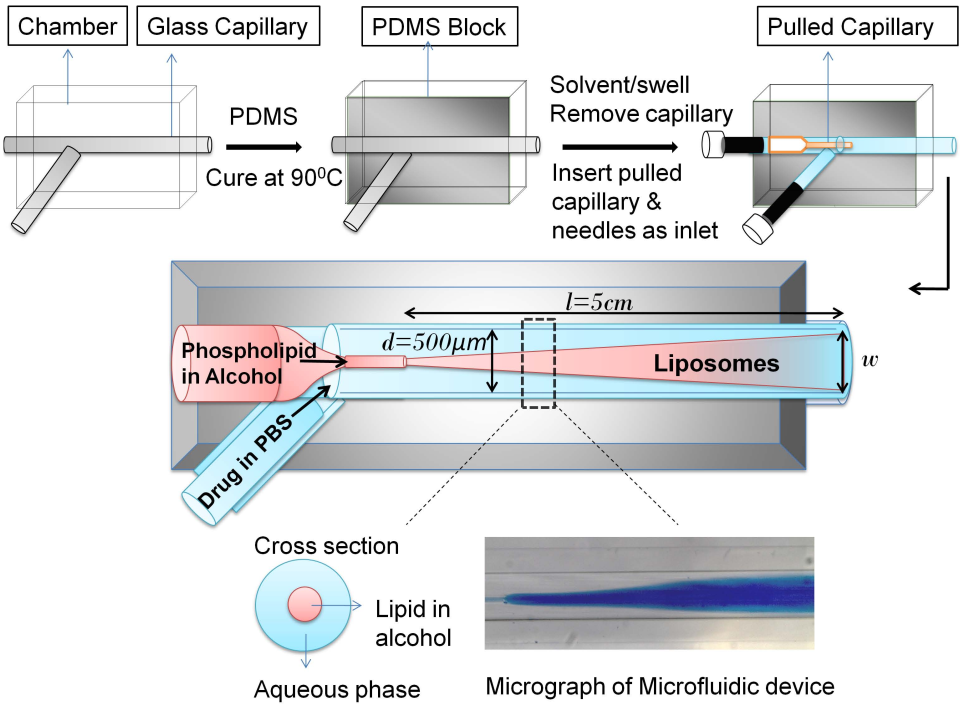

Figure 1: Top: Schematic diagram of core-annular microfluidic device fabrication sequence. Middle: Final assembled configuration of the entire setup. Bottom: Cross section of axial flow focusing and a sample photograph showing the spreading of the dye from the core stream into the annular region. The liposomes are expected to be formed in the mixed region.

New Era pumps Inc.) are used for pumping the fluids inside microchannel.

2.3. *Experimental setup for liposome synthesis*

The vesicles synthesis from the solution phase is only possible above the phase transition temperature (T_g) of the lipid membrane, so it is necessary to maintain a temperature of the system above this. When required, the microfluidic device along with the long teflon tubings are kept inside an incubator with the temperature controlled in the range 27°C to 67°C.

2.4. *Estimation of mean diameter and polydispersity of microfluidic liposomes*

The resultant liposomes are characterized by a single angle back-scatter Dynamic Light Scattering (DLS) or Photon Correlation Spectroscopy (PCS) (Zetasizer nano ZS, Malvern, UK). He-Ne laser at 633 nm wavelength and 4 mW power is used and the scattered light is detected at an angle of 173°. The size and size distribution is calculated using software DTS 6.1 (supplied by Malvern). The Polydispersity and the average liposome diameter are evaluated using cumulant analysis method and the weight-weight distribution of diameter is evaluated using multimodal distribution analysis. Three independent experiments are carried out for liposome synthesis experiments and for each of them, three measurement runs are performed). Ethanol present in the dispersion has a large influence on the viscosity of solution and a negligible effect on the refractive index; these corrections are made using the DTS 6.1 software (Malvern) and were found to be in good agrement with reported values (Song and Peng, 2008).

2.5. *Estimation of size and lamellarity of liposomes*

Tunnelling Electron Microscopy (TEM) (JEM-2100F, Jeol) is performed to check the lamellarity of the liposomes. An electron dense material, 2%w/v phosphotungstic acid (PTA) (SD-Fine chemicals India), is used for negative staining . A drop of the liposome solution (10 μl) is placed carefully on the TEM grid with the help of a micropipette and kept for air drying for 10 min, followed by placing a drop of negative stain. The sample is air dried again before observing at an operating voltage of 120 kV.

Environmental Scanning Electron Microscopy (ESEM) (Quanta-250 FEG, FEI) analysis is performed using a Wet-STEM-detector and the dispersion is observed at a temperature of 4°C 100% humidity, 20 kV accelerating voltage (0.14 nA current), and 840 Pa pressure. Wet STEM allows observation of liposomes keeping its environment fully hydrated i.e. in its native state.

3. Results

In the sections below we first report the observations of various influences on the size and nature of liposomes formed. The co-axial (core-annular) microfluidic device is used for all these experiments, unless otherwise stated. Lipid solution in ethanol is used as the core fluid stream and water is used in the outer stream.

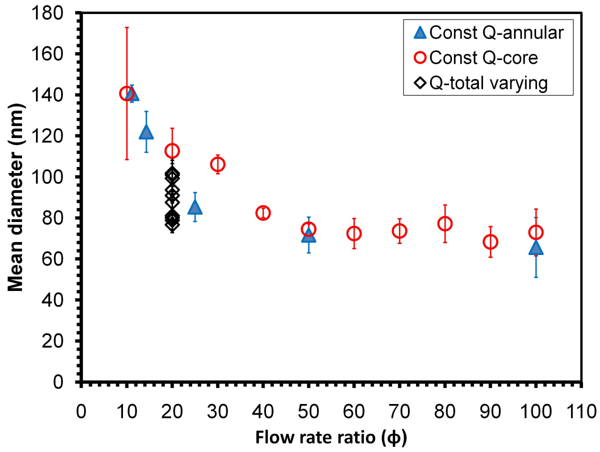

Figure 2: Effect of the flow rate ratio ϕ (outer to inner) and total flow rate on the size of liposome formed. The mean diameter is obtained by DLS. Triangles: Q_W (aqueous in the annular region) is kept constant at 100 μl/min and Q_E (lipidic ethanol in the core region) increases going from right to left. Circles: Q_E is constant at 10 μl/min and Q_W increases from 100 to 1000 μl/min (going from left to right). Diamonds: ϕ = 20 is kept constant but the total flow rate Q is varied from 21 to 945 μl/min, by increasing both the flow rates.

3.1. *Effect of flow on the mean liposome size*

The ratio of the volumetric flow rates of the aqueous (annular) and lipids dissolved in ethanol (core) has been shown to have a significant effect on the size of liposomes in the rectangular cross section geometry (Jahn et al., 2007). We confirm that a similar behaviour is reproducible even in the axial flow focussing geometry. In a later section we also provide a plausible explanation for this behaviour.

The flow rate ratio is denoted here as ϕ, and defined as

$$\phi = \frac{Q_{\text{annular}}}{Q_{\text{core}}} = \frac{Q_W}{Q_E} \tag{1}$$

where Q_{annular} (or Q_W) is the outer (water) volumetric flow rate and Q_{core} (or Q_E) is the inner (ethanol) volumetric flow rate. It is seen from Figure 2 that at lower ϕ, the size decreases with increase in ϕ, and plateaus to a nearly constant value at large ϕ. In this study ϕ has been varied by two ways, one by keeping the inner flow rate constant and other by keeping the outer flow rate constant. The liposome sizes have been determined by PCS (single angle DLS).

It is observed that in both the cases (constant Q_W and constant Q_E), the qualitative behaviour is the same–a decrease followed by a plateau. The large ϕ plateau for the liposome sizes has not been reported in earlier works of microfluidic synthesis (Jahn et al., 2007, 2010), probably owing to the limitation of a channel geometry where obtaining a stable flow is difficult at high flow rate ratios. Though not shown here, in the axial flow focussing we can obtain flow rate ratios as high as ϕ= 500 (Q_E=5 μl/min and Q_W=2500 μl/min).

Whereas the large ϕ plateau of the liposome size is nearly equal in both the cases, there is a variation in the size of liposomes in the two cases for intermediate ϕ around $20 \lessapprox \phi \lessapprox 30$. This suggests that there could be other parameters influencing the size of liposomes, rather than just the flow-rate ratio, as thought previously (Jahn et al., 2007).

The presence of other influences on the size is more clearly seen when the flow rate ratio ϕ is kept constant, while increasing the overall flow rate $Q = Q_W + Q_E$. We show this in Figure 3, where the size of liposomes, at a constant flow rate ratio $\phi = 20$, is plotted against the Reynolds number $\text{Re} = d\,U\,\rho/\mu$, where U is the superficial velocity $U \equiv 4Q/\pi\,d^2$, d is the inner diameter of the outer channel, ρ is the mean mass density and μ is the viscosity of the fluid (here taken as that of water). At low velocities, the size is nearly constant (at a value around 100 nm, comparable to the low ϕ sizes shown in Figure 2), whereas at higher velocities it approaches a value close to 70 nm, close to the large ϕ plateau of Figure 2. This variation is also shown in Figure 2 (as diamonds at constant ϕ = 20). Again this observation has not been reported earlier, possibly because in the regimes explored in Jahn et al. (2007) the overall flow rate was incidentally varied only in the region where it had no influence.

The flow rate ratio ϕ also specifies the ternary component composition (water:ethanol:lipid) in the final collected sample. A lower ϕ implies a higher fraction of lipid and ethanol in the ultimate mixture. The size of the liposomes cannot, therefore, be characterised in a simple ternary component diagram (Ishii et al., 1995) which are commonly used for equilibrium systems.

Increasing the overall flow rate amounts to two obvious influences on the local transport phenomena: increase in the local shear rate and an increase in the Peclet number (that measures the relative importance of axial convection to the radial diffusion of species) defined in this case as:

$$\text{Pe} = \frac{U\,d^2}{\mathcal{D}\,L} \tag{2}$$

where, $\mathcal{D}$ is the diffusivity of a solute and L is a characteristic length along the axial direction. The solute in this case is ethanol, which dissolves and diffuses from the core region into water in the annular region.

The influence of shear rate on the dynamics of self assembly of lipids into disc-like micelles, and further aggregation of these micelles towards larger domain growth and closure to form lipid vesicles, can be one possible influence. However, to the best of our knowledge this has not been studied, and therefore, we summarily leave out exploring this possibility.

Figure 3 indicates that increase in the Peclet number Pe (only U is varied in Eq. (2)) leads to a smaller sized vesicle. The value of Pe for this study is varied from $O(10)$ to $O(100)$. A large $\text{Pe} \gg 1$ implies that the two streams hardly mix by diffusion inside the channel of the device. Mixing of the two streams is essential for the formation liposomes. It is only when water and ethanol inter-diffuse and the lipids can no longer remain in the dispersed form (due to the energetic costs in the presence surrounding water molecules) that they self-assemble to form vesicles. Figure 3 indicates that Pe values are very large for the entire range of flow rates studied, and there is a variation in the liposome sizes. This is counter intuitive. $\text{Pe} \gg 1$ implies the flow inside the channel does not lead to (diffusive) mixing of the streams and therefore does not induce any self assembly. The mixing of the two streams takes place outside the channel

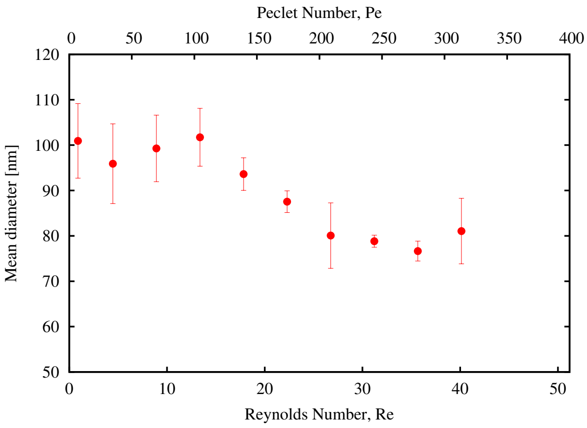

Figure 3: Effect of the total flow rate on the size of liposomes. The Reynolds number Re is shown as one measure of the total flow rate. Also shown is an equivalent axis in terms of the Peclet number Pe. The experiments are conducted at a constant $\phi = 20$, and varying total flow rate Q such that $Q_E = 5, 10, \ldots, 45, 50$ μl/min.

as the outlet flow is collected in a cuvette, in which case the liposome size should have been uniform for all Pe $\gg 1$. This suggests that there could be other phenomena occurring in the channel at Pe ≈ 200 that is inducing an increase in the liposome size.

3.2. *Influence of the type of Mixing*

Though several variants of ethanol injection methods have been employed (Batzri and Korn, 1973; Kremer et al., 1977; Wagner et al., 2002; Ishii et al., 1995), there is no reported analysis of what influences the size of liposomes. Here we employed various types of mixing the fluids and observed the dependence of the liposome size. Figure 4 shows that the liposome size is a function of lipid concentration for various ways of mixing the lipid/ethanol with the aqueous phase: (a) mixing of lipid-ethanol by injection in the aqueous phase under continuous stirring, maintaining the ratio of volumes at 1:10 (equivalent to the flow situation of $\phi = 10$) (b) bulk vigorous mixing of lipid-ethanol phase stream with an aqueous phase stream, each of them pumped using calibrated syringe pumps. Here the flow rate of lipid solution was 10 μl/min and aqueous phase was 100 μl/min, and (c) Mixing by microfluidic device, here again the flow rates were similar as in (b). In each case, the ratios of lipid:ethanol:water are kept constant. It is clear from Figure 4, that direct mixing by turbulence yields smaller vesicles (SUVs), mixing in streams gives slightly larger vesicles and microfluidic mixing gives even larger vesicles. The first two methods showed a dependence of size on the lipid concentration in ethanol but in the case of microfluidic mixing, liposome size seems independent of lipid concentration, for even as high as 80 mg/ml of DMPC. Methods (a) and (b) also show polydispersed population (not shown here).

Though the implication of the effect of concentration is not evident, we can conclude on the effect of nature of mixing on the size of liposomes. The stronger the nature of mixing the smaller the size. In order to form larger liposomes, the bilayer membrane discs has to grow to large sizes before closing upon themselves. However, this growth seems to be hindered by the stronger flow, leading to earlier closure and therefore smaller liposomes.

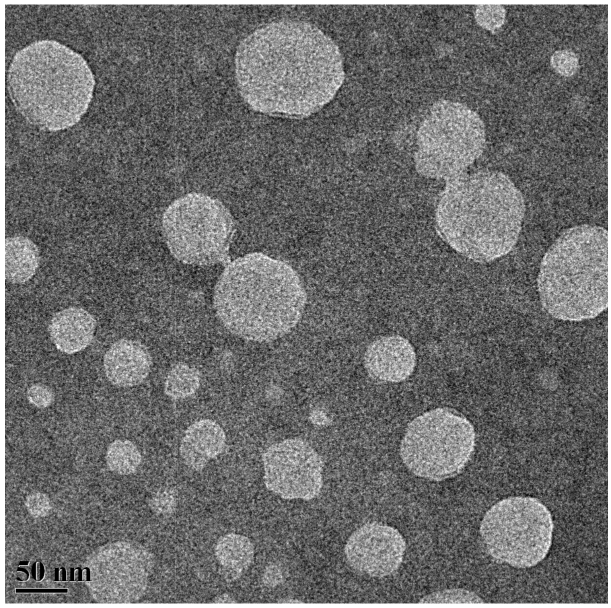

Figure 5: Sample TEM micrographs of DMPC liposomes showing unilamellar membrane liposomes. The synthesis conditions are: 10 mg/ml DMPC, 10 μl/min ethanol flow rate and 200 μl/min water flow rate.

3.3. *Liposome Characterisation*

The liposomes formed by the co-axial flow focussing device have also been characterised by the usual techniques. The particle size distribution by DLS of liposomes is nearly monodisperse with the polydispersity index is around 0.2±0.5. Figure 5 shows a Transmission Electron Microscope (TEM) micrograph of DMPC liposomes, clearly displaying the unilamellar bilayer membranes using the negative staining method used for visualizing. Combined with the near mono-disperse nature of the suspension, this method is well suited to carry calculated amounts drugs. Figure 6 shows the intact and spherical shaped liposome, fairly uniform size distribution of liposome prepared with microfluidic synthesis. Figure 6 also shows a comparison of particle size distribution obtained with DLS along with that obtained by visual analysis of ESEM results, indicating that the size distribution obtained in DLS is a reliable measure of the actual distribution.

3.4. *Effect of lipid concentration*

In coacervation techniques, it is well known that liposome size and polydispersity increases with lipid concentration (Kremer et al., 1977; Wagner et al., 2006). We report the effect of three different DMPC stock concentration–10, 20 and 40 mg/ml of ethanol using the axial flow-focussing device (Figure 7). For 10 and 20 mg/ml of lipids, the concentration does not seem to affect the size of liposomes. The concentration of lipids seems to have an effect only at large values of ϕ. At smaller values of ϕ the concentration of lipids does not significantly influence

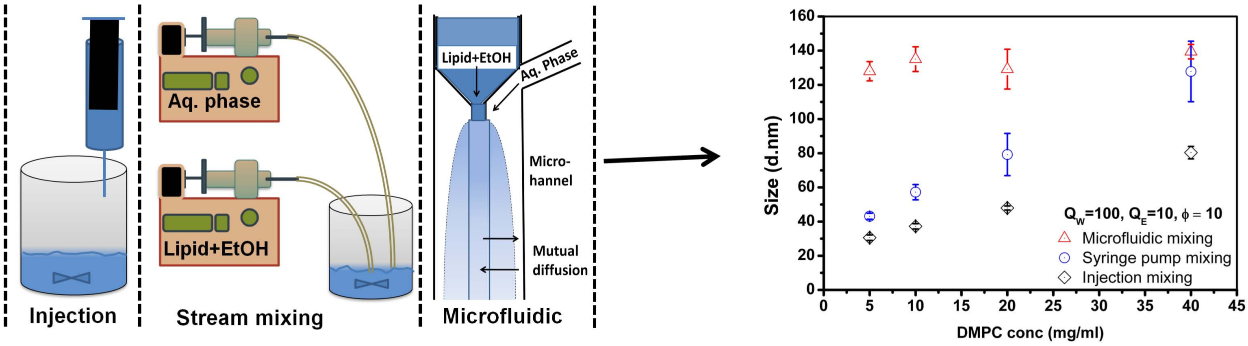

Figure 4: Three types of mixing methods employed to study the effects of bulk mixing: (a) Bulk Injection—by injecting lipid-ethanol in the aqueous phase under continuous stirring; (b) Stream mixing (Syringe Pump)—mixing of lipid-ethanol phase stream with an aqueous phase stream each driven the help of syringe pumps and fine Teflon tubing. The flow rate of lipid solution was 10 μl per min and aqueous phase was kept at 100 μl per min. (c) Laminar Microfluidic mixing. The flow rates were same as in stream mixing. In all the above methods, the volumetric ratio of ethanol to aqueous phase is 1:10. The plot on the right hand side shows the liposome size as a function of concentration of lipids and the type of mixing.

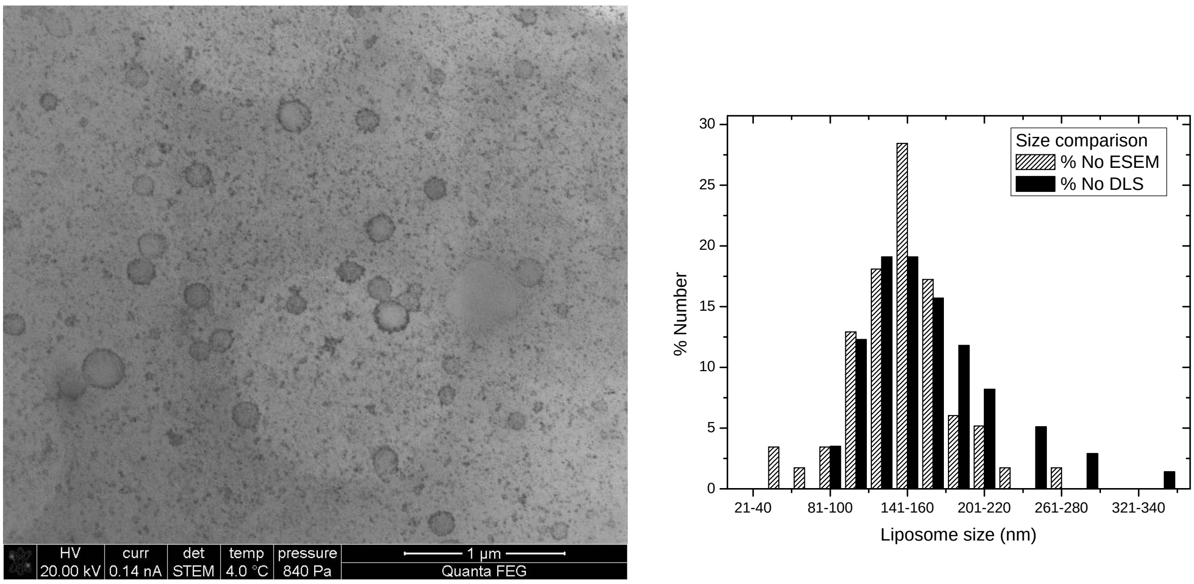

Figure 6: Environmental Scanning Electron Microscopy (ESEM) of HSPC liposomes: Q_E=10 μl/min, Q_W=400 μl/min, ϕ=40. Right: Comparison of number distribution of liposome size obtained by ESEM and DLS.

the size of the liposomes. This also happens to be the region of ϕ where we see a variation in the size upon changes in ϕ. This again indicates that there are other phenomenon that influences self assembly in this region of flow.

3.5. *Effect of membrane composition*

The mean diameter of vesicles is also a function of phospholipid chemistry and membrane composition as seen from Figure 8. To study the effect of cholesterol on liposome size, DMPC:Cholesterol in a ratio 9:1 %w/w is dissolved in ethanol, so that the final lipid concentration in the stock solution is 10 mg/ml. We see that addition of cholesterol increases the liposome size. Addition of cholesterol increases the bending rigidity modulus K_b and area compressibility modulus K_a (Tierney et al., 2005). Increase in K_b increases stiffness (DMPC+Cholesterol K_b = 4.2×10^{-19} J (Evans and Rawicz, 1990)) thus it becomes difficult for a stiffer bilayer disk to bend and form vesicle as compared to more flexible disk (plain DMPC $K_b = 0.56 \times 10^{-19}$ J (Evans and Rawicz, 1990; Zook and Vreeland, 2010)). The rigid bilayer disk curves into vesicles by thermal fluctuations only when they have grown in size above a critical dimension (Leng et al., 2003). Increased stiffness implies the discs need to grow larger ($r > r_c$) before thermal fluctuation can induce a closure.

Secondly, we alter the lipid chain length and saturation by considering other lipids in place of DMPC as shown in Table 1. DOPC produces vesicles larger in size than DMPC lipids. The bending modulus for DOPC is $K_b = 0.85 \times 10^{-19}$ J (Rawicz et al., 2000), which is larger than that for pure DMPC. Similarly DPPC which has a smaller bending modulus $K_b = 0.4 \times 10^{-19}$ J (Lindahl and Edholm, 2000) results in smaller sized liposomes. In general, it is expected that the average diameter of vesicles increases with increase the bending rigidity (Helfrich, 1973).

Table 1: Chain length and saturated bonds for various lipids employed in this work.

Name	n	l=2n+6	Sat.	T_g	Mol Wt
DMPC	4	14	0	23	677.93
DPPC	5	16	0	41	734.04
DOPC	6	18	1	-20	786.11
SoyPC		16:0 (14.9%), 18:0 (3.7%), 18:1 (11.4%), 18:2 (63%), 18:3 (5.3%), Others (1.2%)	4	-1	775.04

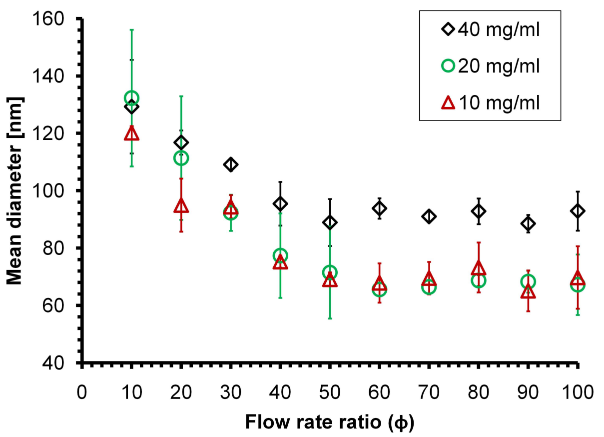

Figure 7: Effect of phospholipid concentration (in ethanol) on the liposome size. At lower ϕ, size is insensitive to the concentration whereas at higher ϕ, the higher concentration of 40 mg/ml produces larger sized liposomes.

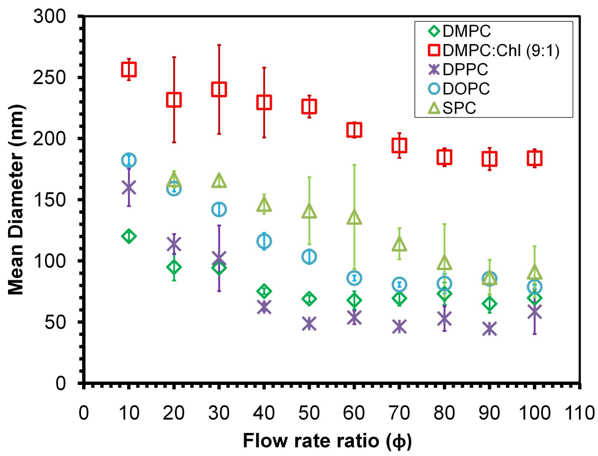

Figure 8: Liposome size as a function of flow rate ratio ϕ and membrane composition.

4. Discussion

4.1. Hypothesis for Liposome Size Variation

The results of the previous section can be summarised as follows (i) there are two regimes of liposome formation: one in which the size depends on the flow parameters but does not depend on the concentration of lipids, and other in which the size is not dependent on flow parameters but is influenced by concentration of lipids. (ii) The dependence on the lipid chemistry and membrane composition is in line with what is expected from a general understanding of the bilayer growth and closure to for vesicles. Therefore, it is the former observation that needs an explanation.

We hypothesise that the two regimes correspond to (a) Convective instability mixing: Instability induced convective mixing within the channel, and (b) Droplet mixing: Mixing of the streams outside the channel when drops of the outlet are collected in a cuvette. Core-annular flows of two miscible fluids where there is a viscosity stratification leads to an instability at large Peclet numbers (Pe $\gg$ 1) and small Reynolds numbers (Re $\equiv d\,U\,\rho/\mu$). This was numerically shown by Ranganathan and Govindarajan (2001) for channel flows and by Selvam et al. (2007) for core-annular flows. In the present case, there is a viscosity variation in a non-monotonic manner, unlike that considered in Selvam et al. (2007) where the viscosity variation is monotonic. d'Olce et al. (2008) have observed certain instabilities experimentally which show pearl and mushroom-like appearance when there is a monotonic increase in the viscosity from the core region to the annular region. To the best of our knowledge such instabilities have not been reported in the water-ethanol system. Nevertheless, we may expect a similar behaviour.

The reason for expecting the droplet mixing regime is that when the Pe number is large and when there is no convective instability, the streams will only mix at the outlet. This is supported by additional facts that the dependence on the concentration of lipids is similar to that seen when two streams are mixed in the various types of ethanol injection methods shown in Figure 4.

At present we do not have a theory to show the possible existence of either of the above claims for the present system. However, it is possible to test the consequences by carrying out visualisation experiments on the same system. In the following we report results of experiments carried out in a similar system—the lipids in ethanol being replaced by a dye—which us allows to visualise the nature of flow inside the device.

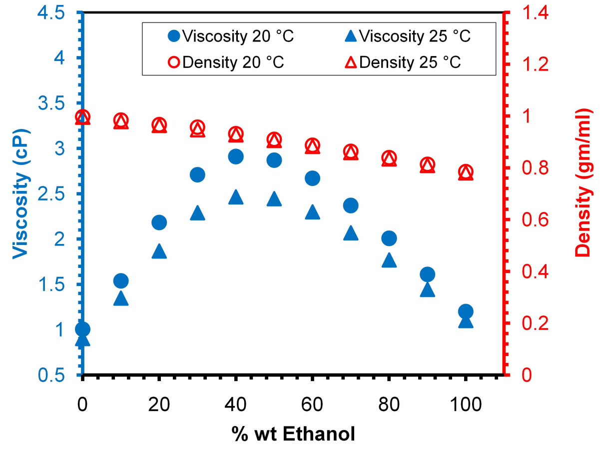

Figure 9: Viscosity (Song and Peng, 2008) and density (Perry et al., 1997) variation of ethanol–water system at different temperatures.

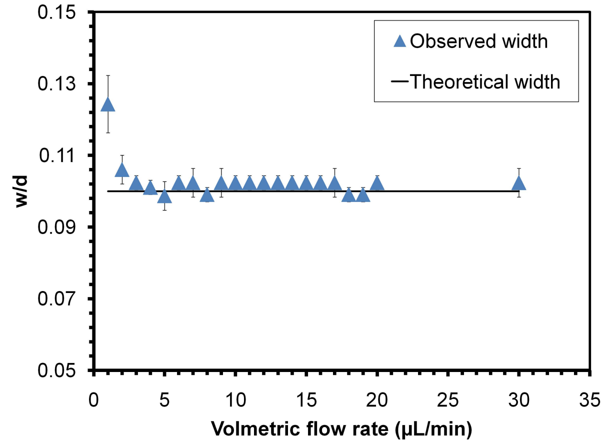

Figure 10: Validation of the width determination. Here the flow rate ratio is kept constant at ϕ=50, increasing the total flow rate.

4.2. *Flow Instability due to Viscosity Stratification*

Core-annular instability is known to occur in immiscible flows. It was shown in Selvam et al. (2007) a similar instability also occurs in miscible fluids, and presence of a small amounts of diffusivity (Pe $\gg$ 1) enhances this instability, particularly at small Reynolds Numbers. Though the model is not identical to the present system, we draw similarities in the nature of flow (core-annular) and the presence of viscosity stratification. In the present case $2 < \mathrm{Re} < 42$ and $16 < \mathrm{Pe} < 507$, based on water-ethanol diffusivity of $\mathcal{D} = 1.28 \times 10^{-}9$ (Zhang et al., 2006).

The variation of viscosity of water-ethanol system (Song and Peng, 2008) is shown in Figure 9. The maximum viscosity of water-ethanol mixture at 20°C is 2.68 mPa.S at 0.7 mole fraction of water; which is around three times the viscosity of water. d'Olce et al. (2008) observed pearl and mushroom like patterns of the instability in the case of a water-natrosol system where the core fluid was of a lower viscosity, and the viscosity increases monotonically to about 25 times the core fluid viscosity. Selvam et al. (2007) also reported instabilities to be present in the opposite case of viscosity decreasing from the core to the annular region. In the present case, we have both the situations. The viscosity is maximum in a small mixed region in between (at about 40%w/w of ethanol), and decreases to about the same value (1 cp) towards core and the periphery.

To study the nature of flow, we carry out the experiments in the absence of lipids, thereby not including the additional complexity of liposome formation. Instead, we introduce a dye—filtered fountain pen ink—to enable visualisation. Experiments were conducted at various flow rate ratios identical to that employed in Figure 2. Since the dye is introduced in the core fluid, we expect that in absence of diffusion of dye, or any other convective instability, the width w of the core (coloured) region in a fully developed flow would be

$$w \approx \frac{d}{\sqrt{2\phi}} \tag{3}$$

This can be obtained by integrating the fully developed Poiseuille flow parabolic velocity profile (assuming uniform viscosity) for the core and annular-fluid volumetric flow rates, and in the limit of $w \ll d$. We validate the experimental method of determining the width of the dye-region (core fluid) which results in the data shown in Figure 10. We choose a large value of $\phi = 50$, and vary the total flow rate. Here, $17 < \mathrm{Pe} < 507$ and $2 < \mathrm{Re} < 64$. As expected, the diffusive mixing should be minimal for large Pe, and the observed width is in agreement with the expected value from Eq. (3).

We have considered a similar set of flow rates as used in the liposome synthesis in Figure 2. In Figure 11 we have considered three variations: (a) Constant flow rate ratio $\phi = 20$ and $\phi = 50$, (b) Varying ϕ keeping outer flow rate constant, and (c) Varying ϕ keeping the inner flow rate constant. At a large flow rate ratio of $\phi = 50$, we see that the width of the core region remains the same.

In Figure 11 (c) we see an unexpected behaviour. The width of the dye region increases with increase in the inner flow rate. We expect that this will decrease ϕ from about 100 to about 10. Though this implies an increase in w from Eq. (3), the increase is much more than what is theoretically expected, as shown in Figure 12. In this set of experiments the Peclet number is high at Pe $\approx$ 35, and increases only marginally from Pe=33 to Pe=36, so this increase in the width cannot be explained even by Taylor diffusion of ethanol into water. Moreover, the dye particles being larger than ethanol, the Peclet number for dye particles is even larger at $\mathrm{Pe}_{\mathrm{dye}} > 10^3$. Therefore, the explanation of the increase in the observed width cannot be given based on mere mass conservation or diffusion, and we conclude that a gentle convective mixing of the fluids takes place due to the growth of an unstable mode. This is a gentle mixing because by nature of the flow, once the fluids mix at low Re the viscosity becomes uniform, which stabilises the flow.

A similar situation is seen when the outer flow rate is varied keeping the inner flow rate constant, as seen in Figure 11 (d) Here too the Peclet number is high, $36 < \mathrm{Pe} < 335$. While we can conclude the existence of a convective instability that

leads to mixing of the fluids, it is difficult to correlate this to the theoretically expected instability value of the Pe or the Re, as the specific model considered in Selvam et al. (2007) is different from the present system.

The mixing of the fluids can be schematically explained with the help of Figure 14. In the stable case with high Pe, the fluid packets move in a laminar fashion, and the diffusion of the particles out of the packet is negligible. Whereas, when the viscosity stratification leads to an instability, the flow does not remain laminar—it mixes the packets of fluids. Here too since the Pe is large, the diffusion of particles out of the packet is negligible on the length scale of the channel diameter, however when seen from a macroscopic perspective, the dye particles occupy a wider region in space as the packets have themselves been dispersed by mixing. To contrast this, a diffusive mixing occurs at a low Peclet and laminar flow, where packets of fluid exchange particles from other packets, purely by diffusion.

4.3. Droplet mixing regime

From the above observations we conclude that not all high Pe and low Re cases leads to an instability. There are regions where there is an agreement of the theoretical width with what is observed. This is summarised in Figure 13, where we see that for very small w there is close agreement, whereas it begins to deviate at w/d around 0.3. In the regime of agreement, the width of the core flow remains nearly constant throughout the length of the channel. At the outlet of the channel, the flow form droplets (the overall flow rates being small). Since the two streams remain unmixed inside the channel, even while forming the drops we observe two visually distinct regions. The fluids only mix when the droplets are gathered in the outlet reservoir.

In Figure 13, the correlation between the liposome size and the width of the dye can be observed. Though these are two different experiments, they have been carried out under the same flow rate conditions. The liposome size is plotted against the observed width, each obtained under the same flow rate values. This plot can be used to infer the nature of flow that would have occurred in the liposome synthesis experiment. When the agreement of the observed width with the theoretical width is good the liposome size is on the lower side, implying the liposome size is governed more by the droplet mixing regime. Moreover, when the observed width deviates from the theory, the liposome size is the largest. This implies that it is the internal convective mixing that is leading to larger size.

4.4. Hypothesis for liposome size variation

Combining the observations of the above experiments with the size of liposomes, we conclude that the liposomes are smaller when the streams mix in the droplet regime, and are larger and when the streams mix by convection inside the channel. The size of liposomes formed in the droplet mixing regime are also comparable with those obtained in the ethanol injection and stream mixing regimes shown in Figure 4.

We hypothesise that the size of the liposomes depends on the nature of convective mixing. More gentle and less turbulent the mixing, the larger the size. In the cases of droplet,

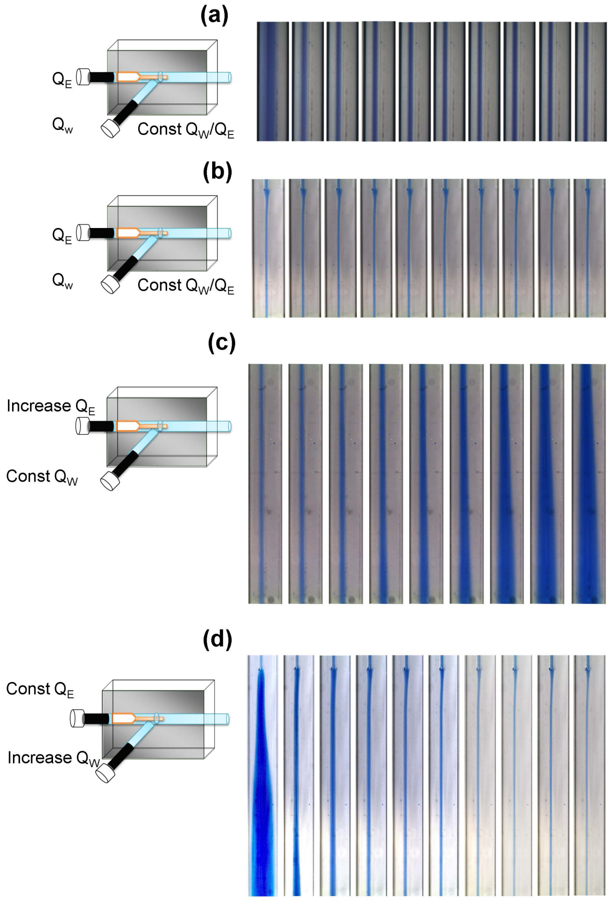

Figure 11: Visualisation of mixing of a blue dyed ethanol (without the lipids) with water, in four typical cases of flow rates employed for liposome synthesis in Figures 2 and 3. (a) Low $\phi = 20$ kept constant, and total flow rate Q is varied such that $Q_E = 5, 10, \ldots, 45, 50$ μl/min, going from left to right. The photographs shows wider region of mixing at lower Q. (b) High $\phi = 50$ kept constant, and total flow rate Q is varied such that $Q_E = 2, 4, \ldots, 16, \&30$ μl/min, going from left to right. The photographs show no mixing of the dye. (c) Constant $Q_W = 100$ μl/min and increasing $Q_E = 1, 2, \ldots, 8, 9$ μl/min showing strong mixing for low ϕ (right most). (d) Constant $Q_E = 5$ μl/min and increasing $Q_W = 100, 200, \ldots, 1000$ μl/min. showing strong mixing at low ϕ (left most).

ethanol injection, and stream mixing, the flow is locally vigorous, whereas in the viscosity stratification led instability mixes the fluids rather gently.

As water mixes with ethanol, the lipids self-assemble when it is no longer favorable to remain in a dispersed form. They begin to assemble as disc-like bilayer membrane micelles (Leng et al., 2003) which grow in size. When the edge energy at the periphery of the discs become comparable to thermal fluctuations induced bending energy of the membrane, the discs close upon to form vesicles (Helfrich, 1973). Left to itself all the vesicles formed would be about the same size as determined by this balance. However, convective motion in the length scales of the discs can induce closure before the membrane grows to larger possible dimensions. It is known that the smallest vesicles are induced by the action of ultrasonic waves, that essentially introduce convective motion in small length scales by spontaneous

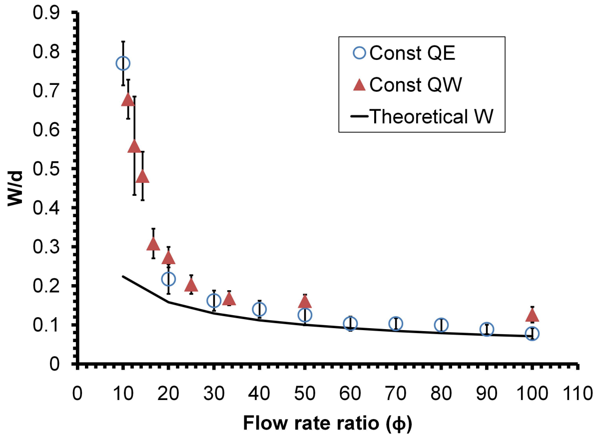

Figure 12: Comparison of the observed spread of the dyed ethanol with near the outlet of the channel, along with the theoretical approximation of the width obtained from Eq. (3).

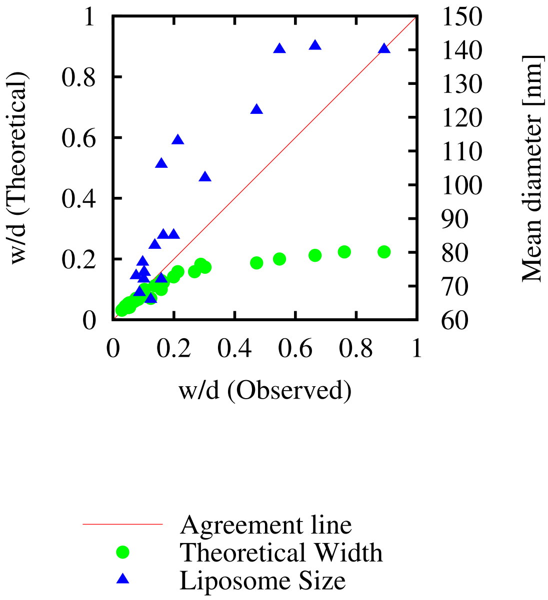

Figure 13: Correlation of the mixing observed visually in Figure 12 with the liposome size obtained under identical flow conditions. Liposome size are smaller when the theoretical width matches with the observed (droplet mixing). Liposomes of largest size are formed when there is a large disagreement in the widths (convective mixing).

vapourisation into bubbles (Richardson et al., 2007; Yamaguchi et al., 2009).

The presence of ethanol is to essentially reduce the interfacial tension along the edges. A reduced tension will lead to a larger sized liposome, since the thermally induced bending of the membrane takes place at a larger size of the disc.

5. Conclusion

Two distinct but connected aspects of microfluidic synthesis of liposomes were shown. The general method employed for liposome synthesis is a variant of the ethanol injection technique in which lipids dissolved in ethanol is contacted with water, leading to the assembly of lipids to closed vesicular structures in the presence of excess water.

The first aspect is a detailed demonstration of a simple device capable to synthesise unilamellar liposomes of nearly uniform size in a co-axial core-annular flow geometry. Detailed observations of the effect of various flow parameters, lipid chemistry and concentration were made. The chief conclusion from these observations are that the size and formation of liposomes cannot be characterised by ternary component diagrams, or just the flow rate ratio ϕ as done in the past; and that the size strongly depends on the nature flow.

The second and more significant aspect is on throwing some light on the hitherto unknown mechanism of liposome size selection in ethanol injection techniques and by implication in a microfluidic setup such as the one employed here. We showed that the size depends strongly on the nature of micro-convective mixing.

In the case of the microfluidic set up, there are two regimes. One in which a convective hydrodynamic instability due to viscosity stratification leads to a gentle mixing and a larger size of liposomes. In the other regime the streams do not mix inside the channel, but mix as droplets formed at the outlet when collected in a reservoir. Vigorous mixing leads to smaller liposomes. Though we do not present a detailed theory for the exact value of size selection, it is argued that it is the length scales of flow (mixing) that determine the size of liposomes.

Further studies into the detailed structure of the flow in microfluidic and other ethanol-injection methods can establish a more accurate connection between the liposome size and the length scales of flow variation. The discovery of this important effect should help in formulating models that will be able to predict liposome size and lamellarity in many coacervation methods.

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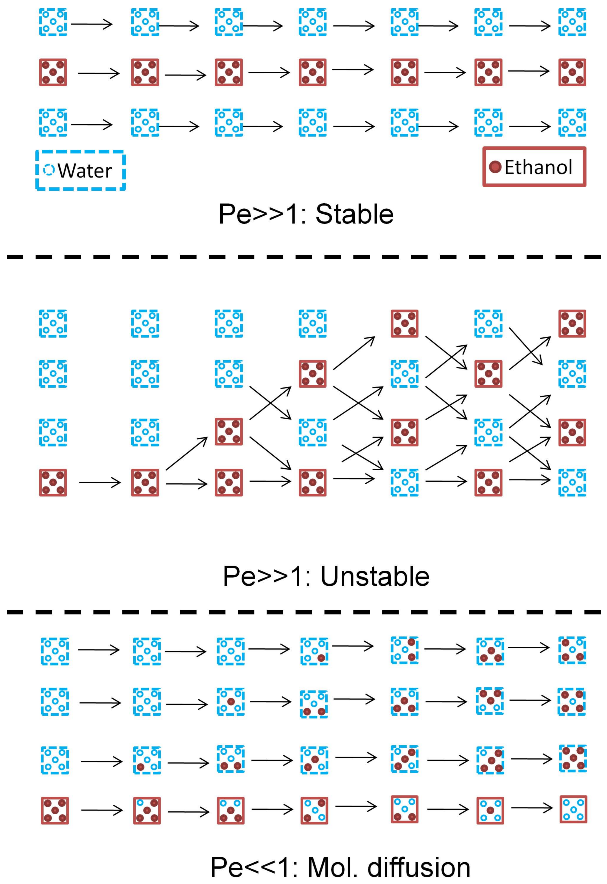

Figure 14: Schematic representation of various forms of mixing observed in Figure 11. Here a box denotes a packet of fluid, and light (blue) circles denote water molecules, and dark (red) circles denote (dyed) ethanol molecules. Top: High Peclet, $\mathrm{Pe} \ll 1$, without any instability the packets traverse in a laminar flow without mixing, as in the case of $\phi = 50$ in Figure 11. Middle: Hydrodynamic instability driven mixing of fluids, still in high $\mathrm{Pe} \ll 1$ implies the particles are still inside the packet of fluid, but the packets themselves mix spreading ethanol (dye). Bottom: In low $\mathrm{Pe} \ll 1$ dominated by diffusion, the spread of ethanol (dye) is due to exchange of particles in the packets, while the packets themselves traverse in a laminar flow.

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